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II. Epitope specific induction of proteinuria by monoclonal antibodies

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II. Epitope specific induction of proteinuria by monoclonal antibodies. A monoclonal antibody, K9/9, directed against a novel epithelial cell surface sialo-glycoprotein, SGP-115/107, present in the rat glomerulus, has been shown to induce glomerular epithelial cell effacement and retraction, and an increase in protein excretion rate upon in vivo administration. Such damage is not seen upon administration of two additional monoclonal antibodies that recognize this epithelial cell antigen, but with different epitope specificities. To further clarify the mechanism of the epithelial cell abnormality, in vitro studies were performed on glomerular epithelial cells established in primary culture. None of these antibodies alone appeared to induce alterations in the cultured cells. However, an antibody of the IgG_{2a} isotype induced complement-dependent cell damage in vitro, although failed to be pathogenic when administered in the intact animal. The pathogenic potential of K9/9 cannot be attributed to its isotype or rates of association or dissociation from the antigen. Studies suggest that all three monoclonal antibodies recognize different, though spatially close epitopes on SGP-115/107. These results demonstrate, for the first time, a complement- and leukocyte-independent mechanism of tissue injury that results from an epitope-specific interaction between a monoclonal antibody and its specific, epithelial cell surface-antigen. Results obtained in other cell systems suggest that abnormalities of epithelial cell structure and function can result from the interaction between specific cell surface components, particularly growth factor receptors, and monoclonal antibodies that mimic the actions of the specific agonist.

Although a number of non-immune mechanisms of glomerular injury appear to play a key role in the progression of established renal diseases in humans and experimental animals [1], the great majority of primary glomerulopathies are caused by an immune mechanism. Such diseases usually involve either exogenous antigens entrapped in the glomerular capillary wall, which subsequently become the target of an appropriately-evoked immune response, or endogenous antigens to which the organism becomes sensitized by an abnormality in the immune-regulatory mechanisms that, under normal circumstances, prevent the development of autoimmunity [2].

In many glomerular diseases, the damage to the glomerular microvasculature occurs through the deposition of antibodies and is mediated either by lytic components of the activated complement system [3, 4], by triggering the coagulation cascade [5], or via inflammatory cells attracted to the glomerular capillary wall by gradients of locally-released chemotactic agents [6, 7]. There remain, however, a certain proportion of glomerulopathies, most notably minimal change disease and closely related conditions, that do not appear to be mediated by any of the above-mentioned systems of tissue injury. The observation of early and persistent recurrent disease that affects allograft recipients in some of the more aggressive forms of these glomerulopathies [8–10] has led investigators to postulate the existence of circulating factors that would mediate or induce the structural and functional glomerular abnormalities. Clearly, other mechanisms, as yet poorly characterized, must be involved in the induction of structural alterations of the glomerular visceral epithelial cell that accompanies the striking permeability changes observed in these disorders. The search for such factors and investigations on potential mechanisms of cell injury by systems other than those summarized above have for the most part remained fruitless.

In this study we describe a novel mechanism of glomerular epithelial cell injury that critically depends on an epitope specific interaction between a cell surface moiety and a monoclonal antibody. The mechanism of cell damage does not involve the classical mediators of immune injury and resembles the changes induced directly in other cell systems by antibodies or specific agonists for cell surface receptors.

Methods

Animals

Female Lewis rats were purchased from Charles River Breeding Laboratories (Wilmington, Massachusetts, USA) and allowed free access to food and water. Female BALB/c mice, eight weeks old, were obtained from Cumberland View Farms (Clinton, Tennessee, USA) and fed ad libitum.

Production and characterization of monoclonal antibodies

Monoclonal antibodies directed at epithelial cell surface antigens were produced as described previously [11]. Class and subclass were determined by double diffusion in agar using commercially prepared antisera (ICN ImmunoBiologicals, Lisle, Illinois, USA). The binding patterns of these monoclonal antibodies to tissues of the rat were determined on 4 μm thick acetone-fixed tissue sections using hybridoma supernatants and fluorescein conjugated, affinity-purified rabbit anti-mouse immunoglobulin as previously described [12].

The ability of these monoclonal antibodies to bind complement in vitro was assessed using an immunofluorescence tech-

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nique on fixed sections of rat kidney [12, 13] and a lytic assay on affinity purified cultures of rat proximal tubule epithelial cells or glomerular epithelial cells as described below.

To obtain large quantities of monoclonal antibodies for in vivo administration to rats, the hybridoma cells were injected into mice previously primed with 2,6,10,14-tetramethylpentadecane (Aldrich Chemical Company, Milwaukee, Wisconsin, USA). The ascites fluid was collected, subjected to a 45% ammonium sulfate precipitation, and the resulting immunoglobulin fraction was dialyzed against PBS for two to three days and against saline for one day. The protein solution was removed from dialysis, subjected to ultrafiltration, and stored at −70°C until used. The antibody preparations were labeled with ^{125}I by the chloramine T technique [13] or with Iodobeads (Pierce Chemical Company, Rockford, Illinois), following the manufacturer's recommendation.

Animal experiments

Normal Lewis rats received monoclonal antibodies contained in 10 to 40 mg of ascitic protein by an intravenous route and a subcutaneous injection of 0.2 ml of Freund's adjuvant containing 5 mg/ml H37 RA *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, Michigan, USA) and 1 mg of ascites derived non-kidney binding immunoglobulin. Rats were housed in metabolic cages with free access to food and water. Urinary protein levels were determined using the sulfosalicylic acid precipitation method [13] with human serum (Lab-Trol, Dade Diagnostics, Aguada, Puerto Rico) serving as protein standards. Differences in urinary excretion within experimental groups was assessed by paired *t*-test for statistical significance. Differences among the three experimental groups were studied by one-way analysis of variance followed by multiple pairwise comparisons.

Animals were sacrificed 24 or 48 hours after antibody administration. The right kidney was removed under Nembutal anesthesia, and coronal sections were placed in 10% formalin or snap frozen in liquid nitrogen. The left kidney was perfused in situ with 1.25% glutaraldehyde and processed for light and electron microscopy as previously described [12]. Frozen sections of the right kidney were processed (unfixed) for direct immunofluorescence microscopy (DIF) using fluorescein conjugated, affinity-purified rabbit anti-mouse immunoglobulin, affinity-purified rabbit anti-rat immunoglobulin, and goat anti-rat C3, as previously described [12]. Formalin fixed portions of the unperfused (right) and perfused (left) kidneys were embedded in paraffin, and 3 μm sections were stained with hematoxylin-eosin or periodic acid-Schiff reagent (PAS) and examined for hypercellularity and structural abnormalities.

The amount of antibody bound in vivo was determined with ^{125}I-labeled immunoglobulin, as described previously [13]. Twenty-four hours after antibody administration, the right kidney was removed under anesthesia and processed for morphologic and immunofluorescence studies as described above. The left kidney was perfused with PBS in situ, removed, and the radioactivity of the tissue measured. The amount of glomerular-bound immunoglobulin was calculated by the method described by Salant, Darby and Couser [14], assuming a total glomerular count of 38,000/kidney.

Antigen and epitope specificities of monoclonal antibodies

The antigen and epitope specificities of the monoclonal antibodies were established by immunoprecipitation, Western blots, electrophoretic analysis of proteolytic peptides, competitive radioimmunoassay on immobilized, proximal tubule brush border vesicles, and by analysis of the association and dissociation characteristics of the antibodies with the antigen.

Immunoprecipitation. For this procedure, glomerular and proximal tubule brush border antigens were solubilized in non-ionic detergent, radiolabeled with ^{125}I, and precipitated with affinity-purified rabbit anti-mouse IgG as described previously [13].

Western blot analysis. Glomerular and brush border antigens were resolved by SDS-polyacrylamide gel electrophoresis. For this purpose, sieve-isolated glomeruli or brush border vesicles were prepared by previously described methods [13]. Then they were solubilized in sample buffer and electrophoresed on 5 to 15% gradient gels by the discontinuous polyacrylamide gel system described by Laemmli [15]. Protein bands were transferred to nitrocellulose paper (Millipore Corp., Bedford, Massachusetts, USA) following the electrophoretic procedure described by Towbin, Staehelin and Gordon [16]. The paper was then cut into 0.8 cm wide strips, and each strip was incubated with a monoclonal antibody. The bound murine immunoglobulin was detected with ^{125}I-labeled affinity purified, rabbit anti-mouse immunoglobulin. Radioactive bands were visualized by direct and indirect autoradiography [13].

Proteolytic peptide analysis. Peptide maps of the antigen recognized by monoclonal antibodies were obtained by a modification of the protease digestion procedure described by Cleveland et al [17]. The iodinated immunoprecipitate was resuspended in 25 μl of 0.4 mg/ml Staph protease (Cooper-Biomedical, Malvern, Pennsylvania, USA) in 0.125 M Tris-HCl buffer, pH 6.8, containing 0.5% SDS and 10% glycerol. The samples were incubated at 37°C for 30 minutes, solubilized by boiling for five minutes in SDS sample buffer, and the supernatant resolved by SDS-polyacrylamide gel electrophoresis on a 15% discontinuous gel system. The gels were stained with Coomassie Blue, destained in methanol/acetic acid, dried, and autoradiographed as described before [13].

Competitive radioimmunoassay. This assay was performed to further characterize the epitope specificity of the monoclonal antibodies. The procedure was done following a modification of the technique described by Ways and Parham [18]. Microtiter plates (Dynatech Laboratories, Alexandria, Virginia, USA) were coated overnight at 4°C with 6 μg/ml of particulate rat brush border membranes in 0.05 M carbonate buffer, pH 9.5. The plates were washed with PBS containing 0.05 ml/100 ml of Tween 20. Seven serial dilutions of blocking antibodies were made in PBS containing 0.5 g/100 ml ovalbumin. The immobilized antigen preparation in each well was then incubated for two hours at room temperature with 75 μl of blocking antibody in decreasing concentrations. At the end of this incubation period, 50 μl of the blocking antibody solution in each well were removed and replaced with 50 μl of the radiolabeled, test-monoclonal antibody preparation diluted in PBS/ovalbumin to an activity of approximately 500,000 cpm/50 μl. The incubation was performed at room temperature for 30 minutes; the plates were washed three times in PBS-Tween, and the wells were cut

out and counted. Control plates were coated with a 1% vol/vol solution of horse serum and incubated with the various dilutions of the blocking monoclonal antibodies and the radiolabeled test-monoclonal antibody. Under these assay conditions, the radiolabeled antibodies did not bind to the control plate. The level of saturation of antigenic sites achieved with the various dilutions of blocking antibodies was determined in additional experiments. Antigen-coated plates were incubated with the various dilutions of blocking antibodies, washed, and incubated with ^{125}I-labeled affinity-purified, rabbit anti-mouse immunoglobulin. By this procedure, all blocking antibodies were shown to saturate the immobilized antigen at least for the three lowest dilutions tested. Antibodies used in these competitive radioimmunoassays included two monoclonal antibodies (K35/9, K35/3) directed against glycoprotein 330 (gp330) of the proximal tubule and glomerular epithelial cell membrane, a non-binding monoclonal antibody (K16/16) which does not recognize renal antigens, and three monoclonal antibodies with specificity for the 115/107 kd epithelial antigen, SGP-115/107. Unlabeled dilutions of the test-monoclonal antibody were used as a positive blocking control.

Association and dissociation measurements. Relative association and dissociation rates for each monoclonal antibody were determined by a modification of the techniques described by Ways and Parham [19] and Mason and Williams [20]. For this purpose, the NP-40-solubilized brush border antigen preparation was immobilized on Sepharose CL-4B (Pharmacia, Inc., Piscataway, New Jersey, USA) following the manufacturer's directions. The monoclonal antibodies were purified from ascites fluid by ammonium sulfate precipitation and labeled with ^{125}I using Iodobeads. Non-specific binding and entrapment of the radiolabeled antibody by the Sepharose was determined in parallel experiments utilizing Sepharose coupled with a non-specific protein (the immunoglobulin fraction of mouse ascites).

Each radiolabeled monoclonal antibody was incubated in microfuge tubes with an excess of Sepharose-bound brush border antigen or the Sepharose-bound control protein. The tubes were incubated on a tube rotator at room temperature for one hour. The Sepharose preparations were pelleted and washed three times in PBS containing 0.1 g/100 ml of sodium azide. During the last wash, the Sepharose suspensions were transferred to new microfuge tubes to eliminate any background binding of radioactive components to the walls of the tubes. The net amount of antibody bound to its target antigen was expressed as the difference between the mean radioactivity bound to the brush border antigen preparation and that bound to the control protein. This amount was considered 100% of bindable counts.

To obtain an estimate of the relative association rates between the antigen and the various monoclonal antibodies, 500 μl of a radiolabeled antibody, containing 500,000 cpm, was incubated on ice with 500 μl of a 40% vol/vol suspension of each Sepharose preparation in duplicate for times ranging from 2 to 14 minutes. To avoid dissociation of the antigen/antibody complexes during repeated wash periods, a single dilution step into 45 ml of cold PBS was performed, followed by centrifugation. Using this washing step, the background binding to the control protein ranged between 1 and 2% of the added antibody. The net amount of antibody bound at various incubation periods was then expressed as a percentage of the value obtained at one hour (considered as 100% bindable counts, see above).

An estimate of the relative dissociation rates was also obtained by a similar technique. For this purpose 1 ml of a 20% vol/vol suspension of each Sepharose preparation was incubated with iodinated monoclonal antibody (approximately 1×10^6 cpm) on a shaker at room temperature for 30 minutes, an incubation time sufficient to achieve steady state binding of the antibody to its target antigen. The Sepharose pellet was washed as described above, transferred to a new microfuge tube and recounted. The percentage of bound counts was calculated for each preparation after subtracting the non-specific binding of the radiolabeled monoclonal antibody to the Sepharose-bound control protein. The tubes were replaced on the shaker at room temperature and incubated for 3, 7, 24, 48, 76, 100, and 124 hours. At the end of each time period, the tubes were centrifuged, the supernatant aspirated, the Sepharose resuspended in PBS/azide, and the tubes counted. All experiments were performed in triplicate. Proteolysis of the immobilized antigen over a 24 hour period at room temperature, as determined by the capacity to bind specific monoclonal antibodies maximally, and spontaneous loss of the antibody activity over a similar period of incubation time at room temperature was negligible.

Cell culture experiments

Rat glomerular epithelial cells were established in primary culture following the technique described by Harper et al [21]. Affinity purified rat, proximal tubule epithelial cells were grown on fibronectin-coated wells of 24-well plates as previously described by us [22]. The relative quantity of mouse immunoglobulin bound to cultured cells was determined by a radioimmunoassay. Cells were incubated for 60 minutes with monoclonal antibodies, washed, and exposed to ^{125}I-labeled affinity-purified, rabbit anti-mouse immunoglobulin for 60 minutes at 4°C. The cells were washed repeatedly, solubilized in detergent, and the specific binding of ^{125}I-rabbit anti-mouse immunoglobulin determined in a Beckman Biogamma II gamma counter (Beckman Instruments, Inc., Irvine, California, USA). The net amount of second antibody bound to the cells was calculated as the difference in radioactivity of cells exposed to the specific monoclonal antibody and cells exposed to a non-binding antibody of the same isotype. All quantitative values were derived from experiments performed in triplicate.

To assess a potential pathogenic role of the monoclonal antibodies under study, cells established in culture for three to seven days were incubated with an antibody in the presence or absence of a source of complement. Cell viability was established by the degree of incorporation of radiolabeled amino acids into cellular proteins. For these studies, cells were washed with Hank's balanced salt solution containing 1% fcs and 10 mM HEPES, pH 7.5, and were incubated at 4°C for 60 minutes in this buffer containing an immunoglobulin fraction of ascitic fluid (final concentration of immunoglobulin = 0.2 to 0.4 mg/ml). After washing, the cells were incubated at 37°C for one hour with normal human serum diluted 1:10 in barbital buffer (0.005 M barbital, 0.142 M NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.35). The cells were then washed and re-incubated for one to three hours at 37°C in leucine-free RPMI 1640 containing 5% fcs, 2 mM L-glutamine, 10 mM HEPES, and 5 μCi/ml tritiated leucine (Amersham Corporation, Arlington Heights, Illinois,

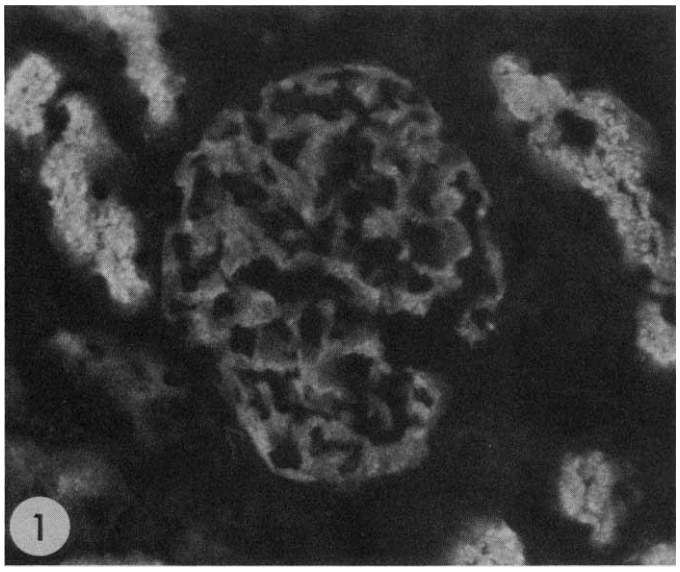

Fig. 1. *Distribution of SGP-115/107 in the renal cortex.* This immunofluorescence micrograph shows the binding pattern seen with monoclonal antibody K9/9. Identical results were obtained with monoclonal antibodies K35/4 and K35/64. The antibody binds to the glomerular capillary wall and to the brush border of the proximal tubules. (Indirect immunofluorescence microscopy on normal rat renal cortex, ×475).

Table 1. Characteristics of monoclonal antibodies

mAb	Antigen specificity *molecular wt*	Class	C-binding in vitro	Proteinuria in vivo
K9/9	GCW, BB; 115/107 kd	IgG_1	−	+
K35/4	GCW, BB; 115/107 kd	IgG_{2a}	+	−
K35/64	GCW, BB; 115/107 kd	IgG_1	−	−
K35/9	gp330; 330 kd	IgG_{2a}	+	−
K35/3	gp330; 330 kd	IgG_1	−	−
K35/31	GEC; ND	IgG_{2a}	+	−
K16/16	negative control	IgG_1	−	−
E1/A	negative control	IgG_{2a}	−	−

Abbreviations are: mAb, monoclonal antibody; GCW, glomerular capillary wall; BB, brush border of proximal tubule epithelium; GEC, glomerular visceral epithelial cells; gp330, glycoprotein of the coated pits and vesicles; ND, not determined.

USA). Cells were washed, solubilized in SDS and the incorporation of tritiated leucine measured in an LKB RackBeta scintillation counter (LKB Instruments, Inc., Gaithersburg, Maryland, USA) utilizing Ecolite scintillation fluid (WestChem, San Diego, California, USA). The inhibition of protein synthesis was calculated using the mean values obtained from six cultures and expressed as a percentage of the values obtained from cells incubated with the same antibody and heat-inactivated human serum. The inhibition of protein synthesis is a sensitive measure of cellular dysfunction which precedes cell death [23, 24] and was utilized in these studies to assess not only cell death resulting from lysis but also to detect an antibody- or complement-dependent sub-lethal effect leading to cellular dysfunction. Control experiments, performed with the trypan blue dye exclusion test [25], confirmed that inhibition of protein synthesis was an objective and sensitive assay of cell injury.

Results

Characterization of monoclonal antibodies

Three monoclonal antibodies with identical immunohistological binding to the rat kidney were studied for their nephrotoxic capacity and antigen/epitope specificity. K9/9 and K35/64 are of the IgG_1 isotype while K35/4 is of the IgG_{2a} subclass. All three antibodies bind in a diffuse fashion to the glomerular capillary wall and along the surface of the proximal tubule brush border (Fig. 1). Reactive antigenic sites are also present on the surface of the intestinal brush border, on the biliary pole of the hepatocyte, the luminal cell surface of bile ducts, and the venules of the spleen, as described previously [13]. There is no detectable difference among these three monoclonal antibodies in their binding characteristics when studied by indirect immunohistochemical techniques. For competitive antibody binding studies on Sepharose-bound brush border antigen, we utilized two additional monoclonal antibodies (K35/3, of the IgG_1 isotype, and K35/9, an IgG_{2a} antibody), with specificity for an antigen present on coated pits and vesicles located at the base of the proximal tubule brush border and focally on glomerular epithelial cells (Table 1). Comparative antibody-binding studies and complement-mediated lysis experiments on cultured cells were performed with K9/9, K35/4, K35/64, and an additional monoclonal antibody specific for a glomerular epithelial cell surface antigen (K35/31, of the IgG_{2a} subclass). Two monoclonal antibodies without specificity for rat tissue antigens (K16/16 and E1/A; Table 1) served as negative controls.

Antigen specificity of monoclonal antibodies

Monoclonal antibodies of K9/9, K35/4, and K35/64 specifically immunoprecipitate two peptide bands with apparent molecular weights of 115 and 107 kilodaltons (kd) from NP-40 solubilized glomerular antigens (SGP-115/107). Only the 107 kd band is immunoprecipitated from an antigen preparation derived from purified renal brush border vesicles (Fig. 2). These findings suggest that the 115 kd peptide is of glomerular origin, while the 107 kd antigen is likely to be derived from the proximal tubule. Contamination of the isolated glomeruli by proximal tubule fragments could account for the presence of the small brush border peptide in the immunoprecipitates derived from glomeruli. Identical proteolytic peptide bands were obtained following protease digestion of the antigen(s) immunoprecipitated by the three monoclonal antibodies (Fig. 3). Collectively, these results and those reported previously [13] indicate that monoclonal antibodies K9/9, K35/4, and K35/64 are specific for a single antigen shared by epithelial cells of various origins, including the glomerulus and the proximal tubule; the small difference in molecular weight between the glomerular- and the brush border-derived component is likely to be due to variations in the degree of glycosylation.

Monoclonal antibodies K35/9 and K35/3 immunoprecipitate a large glycoprotein with apparent molecular weight of about 330 kd (gp330) from the solubilized brush border antigen preparation. The glomerular epithelial cell antigen recognized by K35/31 has not been isolated from detergent-solubilized glomeruli,

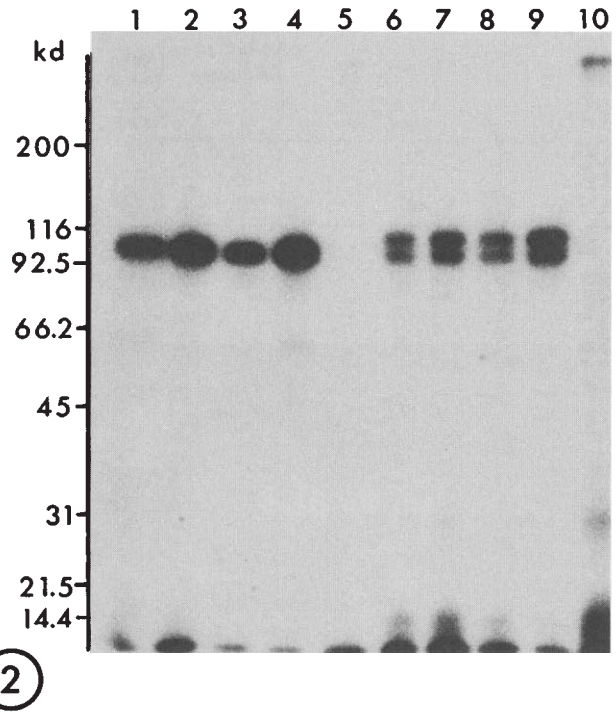

Fig. 2. *Autoradiograph of renal antigens immunoprecipitated from detergent-solubilized brush border (lanes 1 to 5) and glomerular cell membranes (lanes 6 to 10) by monoclonal antibodies.* Iodinated antigens were immunoprecipitated and resolved by gradient SDS electrophoresis under reducing conditions. K9/9, lanes 1, 3, 6, and 8; K35/4, lanes 2 and 7; K35/64, lanes 4 and 9; K16/16 (non-kidney reactive monoclonal antibody), lanes 5 and 10. The three antibodies immunoprecipitate a peptide band with a relative mobility of 107 kd from the brush border antigen preparation while an additional 115 kd component is identified in the immunoprecipitates from the solubilized glomeruli (direct and indirect autoradiography).

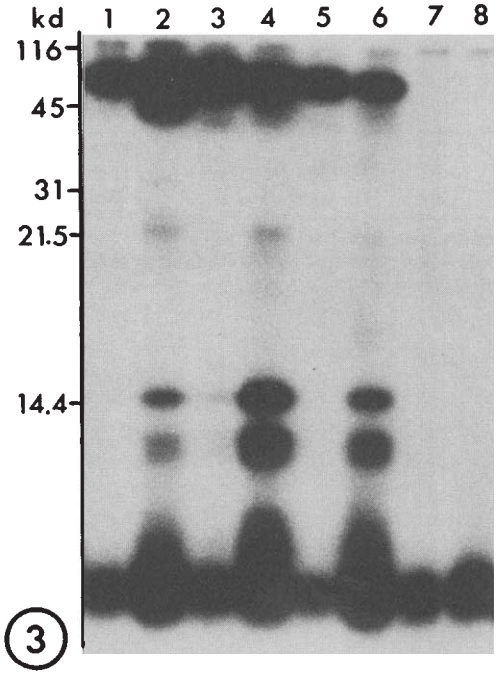

Fig. 3. *Proteolytic peptides of immunoprecipitated proximal tubule brush border antigen resolved by SDS-PAGE on a 15% gel.* The bands seen in lanes 1, 3, 5, and 7 show undigested peptides immunoprecipitated with K9/9, K35/4, K35/64, and control monoclonal antibody, K16/16, respectively. When these immunoprecipitated antigens were subjected to protease digestion (lanes 2, 4, 6, and 8), identical peptide bands were identified for the three monoclonal antibodies, K9/9, K35/4, and K35/64. (Direct and indirect autoradiography).

most likely due to loss of the antigenicity of the epitope under the conditions of immunoprecipitation.

Animal experiments

Twenty-four hours following an intravenous administration of monoclonal antibodies K9/9, K35/4, or K35/64, all animals exhibited mouse immunoglobulin bound to the glomerular capillary wall in a diffuse and irregular pattern. Occasionally, focal binding was also seen along the tubular basement membrane and on the brush border of the proximal convoluted tubule (Fig. 4). Although all three monoclonal antibodies bound in a similar pattern, only the rats which received K9/9 developed proteinuria during the first 48-hour period post-injection (Table 2). Abnormal urinary protein excretion was not seen in any of the animals injected with either of two anti-gp330 antibodies, K35/31, or the two antibodies without specificity for rat tissues, K16/16 and E1/A. Light microscopic examination of the renal tissue of rats injected with any of these monoclonal antibodies failed to demonstrate an inflammatory response. However, ultrastructural examination of glomeruli from rats injected with K9/9 exhibited prominent visceral epithelial cell vacuolar changes, cell surface microvillous formation, effacement of foot processes, and epithelial cell detachment (Fig. 5A). Focal areas of brush border loss were noted in occasional proximal tubule

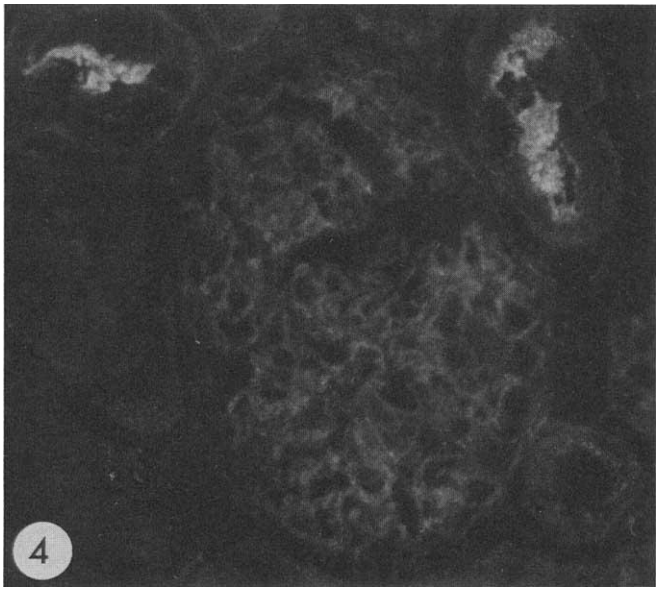

Fig. 4. *Immunofluorescence binding pattern obtained with K9/9 upon in vivo injection of the antibody.* There is weak deposition of the antibody in a diffuse pattern along the glomerular capillary wall. The proximal tubule brush border also shows focal antibody staining. (Direct immunofluorescence microscopy, ×473).

Table 2. Results of animal experiments

mAb Injected	N	Dose mg	Urine protein mg/24 hrs[a] day 0	day 1	day 2
K9/9	18	10	5 ± 2	32 ± 14[b]	28 ± 6[b]
K35/4	6	20–40	5 ± 1	11 ± 7	11 ± 4
K35/64	5	10–40	6 ± 3	9 ± 3	8 ± 2

[a] Values are means ± 1 SD.
[b] $P < 0.05$ when compared to day 0 values or to values seen in groups K35/4 and K35/64

Table 3. Antibody binding studies

Antibody	Dose mg	mAb bound to 2 kidneys μg	mAb bound to glomeruli
K9/9	10	36.5	18
K35/4	15	72.0	23
K35/64	40	258.5	48

The values are the means of two animals in each group.

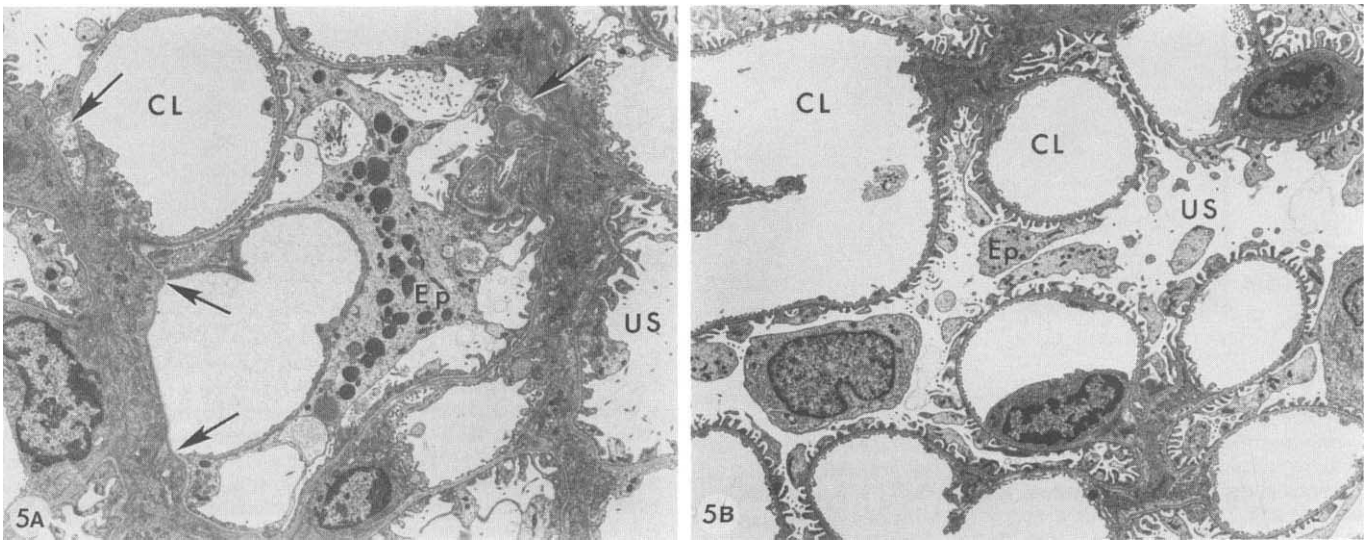

Fig. 5. *Glomerular ultrastructural abnormalities induced in animals injected with monoclonal antibodies directed against the 115/107 kd antigen.* **A.** Animal injected with K9/9. Prominent visceral epithelial cell abnormalities have developed at 24 hours, with increase in lysosomal structures, focal obliteration of the interdigitating architecture of foot processes, surface microvillous formation, and separation of the epithelial cell from the underlying basement membrane (arrows). These changes were associated with an increase in the rate of urinary protein excretion. **B.** Animal injected with K35/4. The ultrastructural details of the glomerular capillary wall are well preserved. Epithelial cells show only minimal microvilli on their cell surface. These animals did not develop proteinuria. CL = capillary lumen; US = urinary space; Ep = visceral epithelial cell. (A ×3500; B ×3150).

profiles. No electron dense deposits were visible along the peripheral glomerular capillary wall, within the mesangial matrix, or along the tubular basement membrane. Animals that received injections of monoclonal antibodies K35/4 or K35/64 exhibited normal glomerular epithelial cell architecture at the ultrastructural level. Focal microvilli were seen only sporadically on glomerular visceral epithelial cells (Fig. 5B). None of the animals exhibited rat C3 bound to renal structures. Glomeruli of animals injected with either the anti-gp330 antibodies (K35/3 and K35/9), the monoclonal antibody with specific binding for glomerular visceral epithelial cells (K35/31), or any of the non-binding control antibodies (K16/16 and E1/A) did not show epithelial cell abnormalities when examined by electron microscopy.

We next sought to determine if the nephrotoxicity of K9/9 and the lack of glomerular injury following administration of K35/4 and K35/64 were related to the amount of immunoglobulin bound within the glomeruli. The in vivo antibody binding studies are summarized in Table 3. Administration of 10 mg of K9/9-containing immunoglobulin fraction to rats was associated with proteinuria with an average of 18 μg of antibody bound to glomerular structures. Conversely, administration of 15 mg of K35/4-containing immunoglobulin or as much as 40 mg of K35/64 did not result in proteinuria, but on average glomerular bound immunoglobulin levels exceeded those seen with K9/9 (Table 3).

Epitope specificities of monoclonal antibodies

Differences in epitope specificity between the three monoclonal antibodies (K9/9, K35/4, and K35/64) directed at SGP-115/107 were established by the results obtained from immunoblot analyses and competitive radioimmunoassays. Of these three monoclonal antibodies, only K9/9 was directed at an epitope on the antigen that remained reactive following SDS denaturation, polyacrylamide gel electrophoresis, and the electrophoretic transblotting. As illustrated in Figure 6, the nitrocellulose strips exposed to K35/4 or K35/64 do not show binding of the ^{125}I-labeled affinity-purified rabbit anti-mouse immunoglobulin. Figure 7 summarizes the results of the competitive radioimmunoassay performed on brush border membrane fragments. As can be seen in Figure 7A, the binding of ^{125}I-labeled K9/9 antibody is completely blocked by an excess of the unlabeled

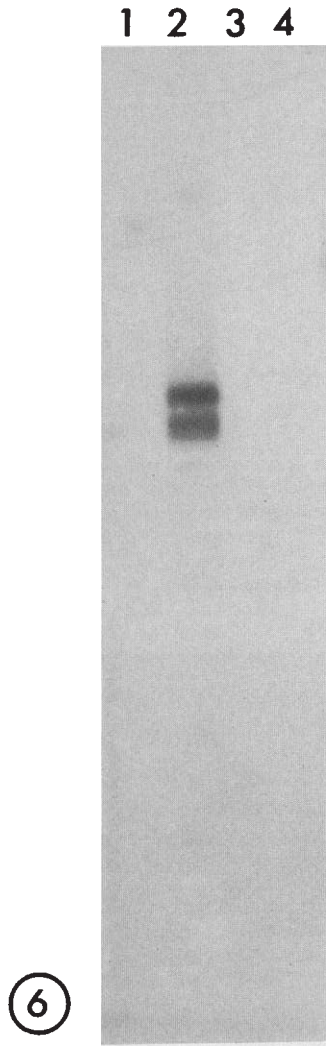

Fig. 6. *Western blot analysis of glomerular proteins reacted with monoclonal antibodies.* Lane 1 illustrates the results obtained on a nitrocellulose strip, following incubation with monoclonal antibody K35/4, lane 2 shows the binding pattern of K9/9. Two peptides with apparent molecular weights of 107 kd and 115 kd are recognized only by K9/9. The SDS-denatured antigen does not bind K35/64 (lane 3). Lane 4 shows the results obtained with the control antibody K16/16. (Direct and indirect autoradiography).

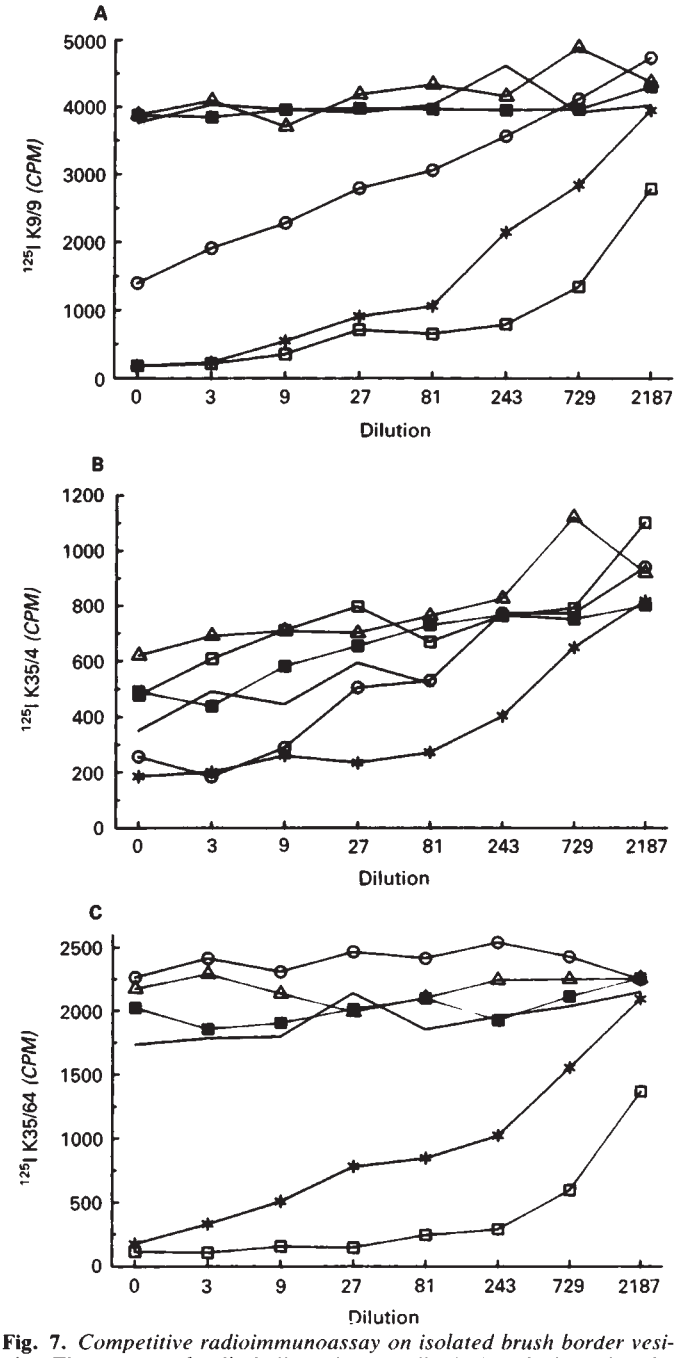

Fig. 7. *Competitive radioimmunoassay on isolated brush border vesicles.* The amount of radio-iodinated test antibody (y-axis) bound to the cell membrane preparation is plotted against the reciprocal value of the dilutions of monoclonal antibodies (x-axis) used to block the binding to the antigen. Three antibodies against the SGP-115/107 antigen (K9/9, asterisks; K35/4, open circles; K35/64, open squares), two antibodies specific for gp330 (K35/9, open triangles; and K35/3, solid squares), and the non-binding monoclonal antibody K16/16 (no marker) were utilized in separate experiments to inhibit binding of ^{125}I-K9/9 (**A**), ^{125}I-K35/4 (**B**), and ^{125}I-K35/64 (**C**).

antibody and by K35/64; only partial blocking is accomplished with an excess of K35/4. No inhibition of binding is observed with anti-gp330 monoclonal antibodies K35/3 and K35/9, or with the control antibody without specificity for rat antigens, K16/16. The radiolabeled K35/4 (Fig. 7B) shows inhibition of binding by the unlabeled antibody preparation and by K9/9, but not by K35/64. Finally, the radiolabeled K35/64 (Fig. 7C) is blocked from binding to its antigenic site by K9/9 and by the unlabeled K35/64, but not by K35/4. None of these antibodies is blocked by the binding of K35/3, K35/9, or K16/16. Taken together, the results seen by the Western blot analyses and those obtained by the blocking immunoassays suggest that K9/9 recognizes a distinct and unique epitope located spatially between the antigenic sites recognized by K35/4 and K35/64.

Properties of the antibodies

To determine if the ability of these monoclonal antibodies to induce glomerular disease was related to their binding charac-

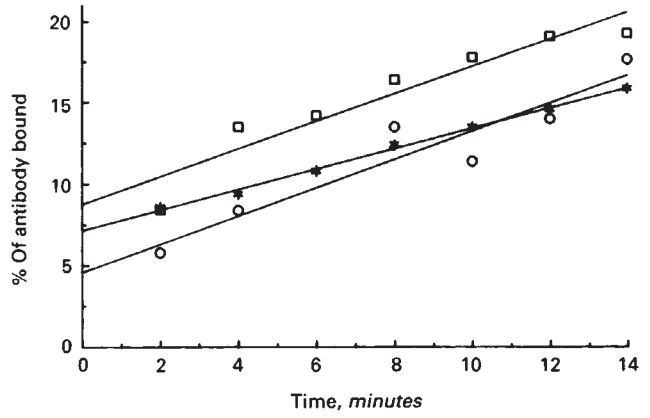

Fig. 8. *Rate of binding of monoclonal antibodies to solubilized brush border proteins immobilized on Sepharose beads.* Lines of regression were drawn through the points obtained for each monoclonal antibody. K35/4 (open circles) and K35/64 (open squares) exhibit similar association rates, as suggested by equivalent slopes of the regression lines, while K9/9 (asterisks) displays a reduced rate of association with the antigen.

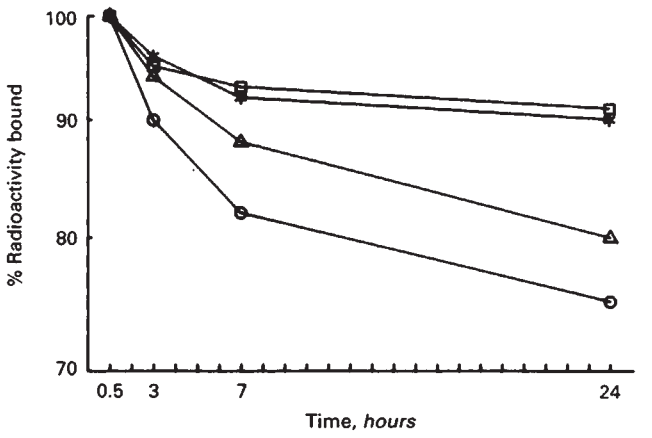

Fig. 9. *Dissociation of monoclonal antibodies from solubilized brush border proteins coupled to Sepharose beads.* The plotted values are expressed as a percentage of the amount bound at thirty minutes. K9/9 (asterisks) and K35/64 (open squares) exhibit similar rates of decline while K35/4 (open circles) and anti-gp330 antibody, K35/9 (open triangles), exhibit faster dissociation rates.

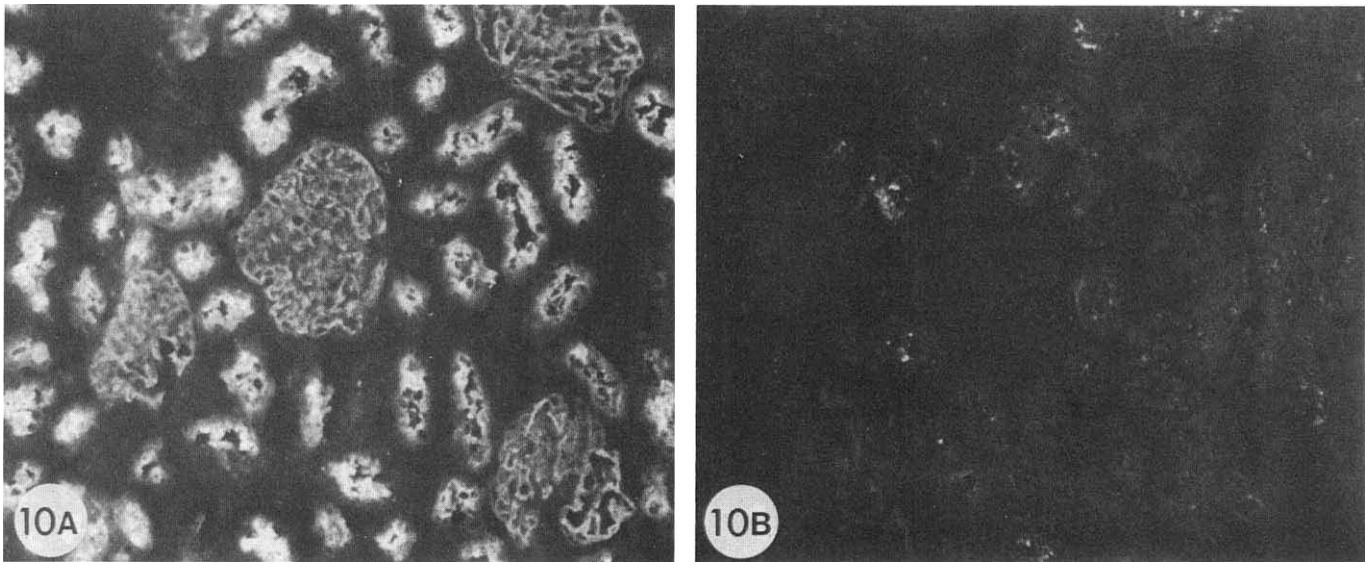

Fig. 10. *In vitro complement-binding capacity of monoclonal antibodies revealed on frozen sections of renal cortex.* **A.** The pattern of human C3 deposition following pre-incubation with K35/4. Complement binding reproduces the distribution of the antigen present within the glomerular capillary wall and on the brush border of the proximal tubules. **B.** The results obtained on the sections pre-incubated with K9/9. The minor amount of C3 bound to tubule profiles was also present in all control slides. Similar results were obtained with monoclonal antibody K35/64 (×190).

teristics, relative rates of association and dissociation from a Sepharose-bound antigen preparation were estimated. The results obtained from such analyses are shown in Figures 8 and 9. Both K35/64 and K35/4 exhibit similar or greater rates of association with the antigen when compared to K9/9, as judged from the slopes of the regression lines that summarize the amount of antibody bound over time. The dissociation from the antigen is somewhat slower over the first 24 hours for K9/9 and K35/64, while K35/4 dissociates more rapidly (Fig. 9). The differences in the dissociation rates were maintained for the various antibodies over six days. The amount of antibody bound to the antigen at 144 hours for K35/64 corresponds to 73% of the amount measured at the beginning of the dissociation assay; for K9/9 the value is 61%, and for K35/4, 41% remains bound at the end of the experiment.

To correlate the complement-binding capacity of these monoclonal antibodies and their in vivo nephrotoxicity, in vitro tests on frozen tissue sections and on cultured glomerular epithelial cell explants were performed. Following in vitro incubation of the three monoclonal antibodies directed at SGP-115/107 on tissue sections, we found that only K35/4 (an IgG_{2a} isotype) was capable of binding human C3 at the sites of antibody deposition (Fig. 10, Table 1). However, this antibody did not induce significant glomerular epithelial cell alterations or proteinuria in

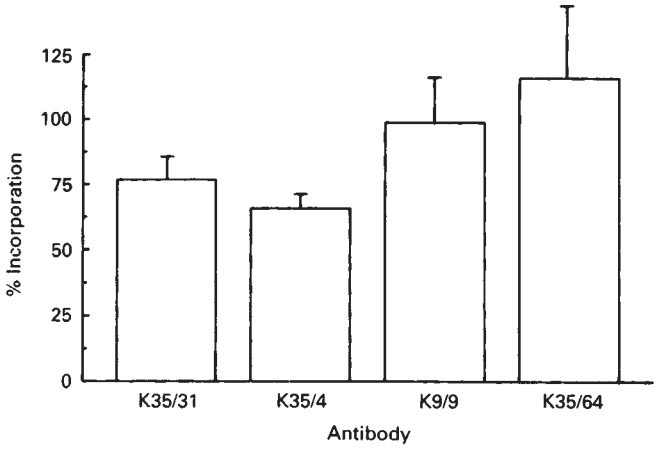

Fig. 11. *Antibody-directed and complement-mediated cell injury of glomerular epithelial cells in culture.* The degree of cell damage was assessed by the decline in the incorporation of ^{3}H-leucine when compared to cells pre-incubated with the antibody and heat-inactivated serum (100% incorporation). The bars represent average values obtained in six culture dishes (± SEM). Monoclonal antibody K35/31 is of the IgG_{2a} subclass, directed at a cell surface antigen present exclusively on glomerular epithelial cells. K35/4 (IgG_{2a}), K9/9 (IgG_1), and K35/64 (IgG_1) are specific for the 115/107 kd epithelial cell antigen.

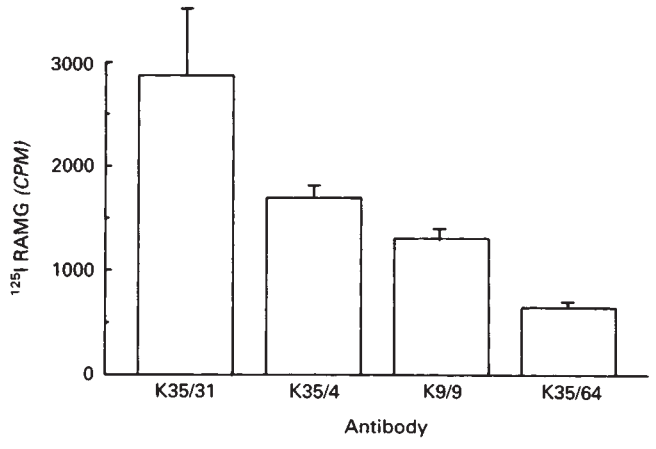

Fig. 12. *Antibody-binding to glomerular epithelial cells in culture.* Cells were incubated with a monoclonal antibody for 1 hour, washed, and then incubated for one hour with ^{125}I affinity purified, rabbit anti-mouse immunoglobulin. Nonspecific binding was determined for unreactive monoclonal antibodies of IgG_1 and IgG_{2a} isotypes and subtracted from the isotype-matched experimental values. Bars represent average values obtained in triplicate (± SEM).

the experimental animal. The other two monoclonal antibodies of the IgG_{2a} isotype, K35/9 and K35/31, with specificities for gp330 and for an unrelated glomerular epithelial cell antigen, respectively, also revealed in vitro complement binding capacity but did not show nephrotoxicity in the intact animal.

A direct effect of K9/9 on cultures of glomerular epithelial cells was not detected by direct microscopic observation. When such cultures were exposed successively to an antibody and a source of complement, only K35/4 and K35/31 produced cellular dysfunction that led to a reduction in the rate of ^{3}H-leucine incorporation (Fig. 11). The rate of amino acid incorporation was reduced to 66% for K35/4 and to 77% for K35/31 when compared to the values obtained with heat-inactivated serum (100%). Monoclonal antibodies K9/9 and K35/64 did not induce cell damage as evidenced by the rate of amino acid incorporation. The relative amounts of immunoglobulin bound to the cells, determined by a radioimmunoassay, are illustrated in Figure 12. As depicted in Figures 11 and 12, the amount of antibody bound to the cells alone did not correlate with the degree of metabolic dysfunction.

Discussion

We have previously reported on the specific interaction of the non-complement binding monoclonal antibody (K9/9) with its specific antigen in the glomerular capillary wall of the rat. The antigenic complex is found in several epithelia and has an apparent molecular weight of 115/107 kd [13]. In the experimental animal, this interaction results in glomerular dysfunction characterized by glomerular visceral epithelial cell effacement and proteinuria. The functional abnormality is elicited only when complete Freund's adjuvant is administered concurrently with an intravenous injection of the antibody. The structural and functional changes are antigen specific, since antibodies deposited in similar quantities in the matrix of the basement membrane (anti-laminin antibodies) or on endothelial and epithelial cells (antibodies directed against a 129/117 kd cell surface component) do not induce such abnormalities [13]. In the present study, we demonstrate that this antibody-induced dysfunction is epitope-specific.

Of three monoclonal antibodies specific for the 115/107 kd antigen, only one, K9/9, induces morphological and functional glomerular alterations in vivo. The differences in immunochemical characteristics among the three antibodies, specifically, the capacity to activate complement and the rate of association and dissociation from the antigen, do not offer a rational explanation for the lack of nephrotoxicity of the two monoclonal antibodies, K35/4 and K35/64. Both these monoclonal antibodies exhibit a somewhat faster association rate than K9/9, while the relative dissociation rates of K9/9 and K35/64 are similar and somewhat slower than that seen for K35/4. Therefore, an unequivocal nexus between nephrotoxicity and antibody affinity is highly improbable. Similar conclusions were reached by Gomez and Richman [26], who reported that the pathogenic potential of monoclonal antibodies directed at the acetylcholine receptor was not proportional to the avidity of the antibody for the antigen in an experimental model of myasthenia gravis.

The glomerular injury induced in vivo by K9/9 is not mediated by complement activation since this antibody does not activate this system on tissue sections of renal cortex or following binding to cultured glomerular or proximal tubule epithelial cells. Deposition of complement components did not occur in the experimental animals injected with K9/9 when examined by direct immunofluorescence microscopy. In contrast, K35/4, of the IgG_{2a} subclass, was capable of mediating complement-dependent lysis of established cultures of glomerular and proximal tubule epithelial cells, yet this monoclonal antibody did not induce significant cellular alterations upon binding to the glomerular capillary wall in vivo. Rat C3 was not demonstrable in the kidneys of rats injected with K35/4. This finding would suggest either a lack of complement activation capacity of this antibody in the intact animal, a rather unlikely possibility in light of our in vitro results, or in vivo activation of

complement components in amounts insufficient to be detected by direct immunofluorescence microscopy. The inability of this complement-binding monoclonal antibody to induce glomerular changes in the experimental animal further emphasizes the unique nature of the interaction between K9/9 and its specific cell surface antigenic site. These findings suggest that the mechanism underlying the epithelial cell abnormality induced by K9/9 could be of critical relevance in the pathogenesis of proteinuric conditions, since it appears to be significantly more effective and sensitive in vivo for disease induction than the antibody-directed (K35/4) and possibly complement-mediated injury involving the same antigen. Such a complement-mediated mechanism of damage of the glomerular epithelial cell has clearly been established in other models of immune complex glomerular diseases [3, 4].

The non-nephritogenic monoclonal antibodies K35/4 and K35/64 recognize different epitopes on the cell surface antigens, as determined by competitive radioimmunoassays and by immunoblots. These two antibody preparations do not induce glomerular injury upon in vivo administration, even though the level of glomerular bound immunoglobulin exceeded that seen in proteinuric animals injected with K9/9. We hypothesize that the epitopes recognized by K35/4 and K35/64 are adjacent to and flank the site recognized by K9/9, since there is reciprocal blocking between K9/9 and K35/4 and between K9/9 and K35/64, but there is no blocking effect apparent between K35/4 and K35/64. Moreover, only K9/9 binds to the denatured protein on Western blots following SDS-polyacrylamide gel electrophoresis of the solubilized cell membrane preparation, a finding that favors the idea that K9/9 recognizes a distinct antigenic site on the protein.

The exact cellular mechanism responsible for the glomerular alterations induced by monoclonal antibody K9/9 in vivo remains unknown. A direct interference of the monoclonal antibody with a cell surface component involved in cell-to-cell or cell-to-matrix contact could account for the changes described above. Imhof et al [27, 28] described perturbations of cell-to-cell adhesion secondary to a loss of tight junctions, with deletion of cell polarity, uncoupling of adjoining cells, changes in cell shape, and elaboration of cell surface microvilli in epithelial cell monolayers derived from canine kidneys (MDCK cells) upon exposure to a unique monoclonal antibody that recognizes a 130/40 kd protein. This antigen, which appears to be related to uvomorulin [28], is normally concentrated at the level of the cell junctions, but it has also been detected on the apical surface in cultured cells before they reach confluence. A mechanism involving perturbation of cell-to-cell contacts by the antibody is less likely to have caused the glomerular epithelial cell changes following K9/9 binding in our animal experiments, given the uniform distribution of the antigen on the luminal side of epithelial cells rather than a more discrete localization to the site of cell contacts, as has been demonstrated for uvomorulin. Epitope specific interactions are also known to occur between cells of different origin, and functional and non-functional sites on molecules involved in lymphocyte adhesion to endothelium and fibroblasts have recently been identified by epitope-specific monoclonal antibodies [29, 30].

A direct effect on glomerular epithelial cell function could also have resulted from conformation changes of a cell surface protein following the binding of an antibody. Histocompatibility antigens are known to undergo three-dimensional rearrangement after the binding of monoclonal antibodies. Such a "structural adaptability" may endow these molecules with the "functional versatility" necessary to interact with many different antigens and T cell receptors [31]. Likewise, investigators working with the HPB-ALL tumor cell line [32] showed that the binding of monoclonal antibodies to the CD3/Ti complex, associated with the T cell receptor, induced an epitope specific increase in intracellular calcium. Changes in calcium influx precede T cell activation, and are thought to occur as the result of a conformational change of the extracellular component of the T cell receptor following the binding of antigen or a specific antibody for the receptor.

The very unique epitope-specificity of the glomerular epithelial cell alterations seen with K9/9 suggests that the antibody might be binding to the active site of a receptor, an enzyme, or a molecule involved in transcellular solute exchange. Cell changes resulting from interactions between monoclonal antibodies and cell surface receptors are not unprecedented. Schreiber et al [33] described cell shape changes in the epithelial cell line A431 exposed to a monoclonal antibody directed against the 170 kd epidermal growth factor (EGF) receptor. The authors reported changes induced by the monoclonal antibody that mimic, in part, the effects of the growth factor on A431 cells in culture [34, 35]. Similarly, agonist-like effects have been reported by Baldwin, Terris and Steiner [36] for anti-insulin receptor antibodies. Since a close structural, biochemical, and functional association of the EGF receptor with the elements of the cytoskeleton has been demonstrated [37–39], growth factor-induced alterations in cell structure and dynamics via changes in the organization of membrane-associated microfilaments are likely to occur. The possibility that SGP-115/107 represents a cell surface enzyme or a transport protein cannot be ruled out. However, the organ and tissue distribution of the antigen and its molecular weight do not correspond to any cell surface component with an established cell function [40–47].

It is conceivable that the 115/107 kd antigen, described by us in the present communication, is a receptor for a growth factor and that the monoclonal antibody K9/9 mimics in part some of the effects of such a factor on the glomerular visceral epithelial cell. In this regard, it is appropriate to note that experimental and human glomerulopathies that exhibit simplification and retraction of foot processes are conditions in which toxic [48–52] or complement-induced epithelial cell damage occur [3, 4]. In such situations, factors that induce cell replication and growth would probably be produced systemically or released locally by damaged cells, and the epithelial cells are likely to be stimulated to regenerate. Furthermore, podocyte simplification and retraction has been observed invariably during nephron hypertrophy secondary to subtotal loss of functioning renal parenchyma [1, 53] in which a causal agent of direct epithelial cell injury is not apparent. Therefore, it is tempting to speculate that an increase in growth-stimulating activity may be a common factor present in all glomerulopathies that display morphological alterations of the visceral epithelium that results in an increased protein excretion rate. Glomerular podocyte retraction and simplification may result as a direct or indirect consequence of such a stimulus on this highly differentiated epithelial cell which is known to have retained only a very limited capacity for cell replication [54, 55]. A monoclonal antibody

directed at the ligand binding site of such a growth factor can be anticipated to induce some of the effects of the agonist as described for other cell systems.

Acknowledgments

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